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Activity of lenalidomide in mantle cell lymphoma can be explained by NK-cell mediated cytotoxicity.

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**Summary:** Lenalidomide is an immunomodulatory agent that has demonstrated clinical benefit for patients with relapsed or refractory mantle cell lymphoma (MCL); however, despite this observed clinical activity, the mechanism of action (MOA) of lenalidomide has not been characterized in this setting. We investigated the MOA of lenalidomide in clinical samples from patients enrolled in the CC-5013-MCL-002 (ClinicalTrials.gov: NCT00875667) trial comparing single-agent lenalidomide vs investigator's choice single-agent therapy and validated our findings in pre-clinical models of MCL. Our results reveal a significant increase in NK-cells relative to total lymphocytes in lenalidomide responders compared to non-responders that was associated with a trend towards prolonged progression free survival and overall survival. Clinical response to lenalidomide was independent of baseline tumor microenvironment expression of its molecular target cereblon, as well as genetic mutations reported to impact clinical response to the Bruton's tyrosine kinase inhibitor ibrutinib. Preclinical experiments revealed lenalidomide enhanced NK cell mediated cytotoxicity against MCL cells via increased lytic immunological synapse formation and secretion of granzyme B. In contrast, lenalidomide exhibited minimal direct cytotoxic effects against MCL cells. Taken together, these data provide the first insight into the clinical activity of lenalidomide against MCL revealing a predominately immune mediated MOA.

**Key words:** lenalidomide, mantle cell lymphoma, natural killer cell, immunomodulation, antibody dependent cell mediated cytotoxicity

**Introduction:**

Mantle cell lymphoma (MCL) is a rare and aggressive histological subtype representing approximately 2% to 7% of non-Hodgkin's lymphoma (NHL) with a median overall survival of approximately 4 years (M. Dreyling, Weigert, Hiddemann, & European, 2008). While the introduction of high dose chemotherapy (with and without autologous stem cell transplantation) and the addition of the anti-CD20 monoclonal antibody rituximab to existing regimens has prolonged progression free survival in front line subjects, ultimately the disease remains incurable (Delarue et al., 2012; Gianni et al., 2003; Romaguera et al., 2005). Despite the availability of agents such as bortezomib, temsirolimus and ibrutinib, the median overall survival remains poor (less than 2 years) in patients with relapsed or refractory MCL (R/R MCL) (Martin Dreyling et al., 2015; Wang et al., 2013). Lenalidomide is an immunomodulatory agent that has demonstrated significant clinical benefit for patients with R/R MCL and is approved for patients who have received at least 2 prior therapies, including the proteasome inhibitor bortezomib (Goy et al., 2013; Habermann et al., 2009; Wiernik et al., 2008; Witzig et al., 2011; Zinzani et al., 2013). Given the availability of treatment options for R/R MCL and the lack of consensus regarding a standard of care in this setting, the phase 2 MCL-002 (SPRINT) study was designed to evaluate single-agent lenalidomide compared to investigator's choice single-agent therapy including rituximab, gemcitabine, fludarabine, chlorambucil, or cytarabine (Trněný et al., 2016). In MCL-002, lenalidomide monotherapy resulted in significant improvements in progression free survival (PFS) [8.7 months] and rates of objective [68%], complete and unconfirmed complete response [5%] compared to investigator's choice [5.2 months PFS; 9% ORR; 0% CR/Cru, respectively], providing the first direct comparison of lenalidomide to other treatment options for R/R MCL (Trněný et al., 2016).

Lenalidomide and other IMiD<sup>®</sup> immunomodulatory agents exhibit a dual mechanism of action, consisting of direct cytotoxic effects against tumor cells as well as targeting the tumor

microenvironment through immunomodulation of T and NK cell activity (Davies et al., 2001; Gandhi et al., 2014; Offidani et al., 2014; Schafer et al., 2003; Wu et al., 2008; Yang et al., 2012; L. H. Zhang et al., 2013). In NHL, the immunomodulatory effects of lenalidomide include repair of dysfunctional T-cell immune synapses in follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL) (Ramsay et al., 2009; Ramsay et al., 2008). Tumor-infiltrating T cells from patients with FL had reduced formation of immune synapses compared to cells from healthy donors; however, treatment with lenalidomide reversed this by enhancing immune synapse formation. Lenalidomide also enhanced rituximab antibody-dependent cellular cytotoxicity (ADCC) in pre-clinical NHL models which provided pre-clinical support for investigation of lenalidomide in combination with rituximab combination regimens for patients with NHL (Gribben, Fowler, & Morschhauser, 2015; Hernandez-Ilizaliturri, Reddy, Holkova, Ottman, & Czuczman, 2005; Reddy et al., 2008; Wu et al., 2008; L. Zhang et al., 2009).

Lenalidomide directly binds Cereblon, a substrate receptor for the CRL4<sup>CRBN</sup> ubiquitin E3-ligase complex that includes damage-specific DNA binding protein (DDB1), cullin 4A (Cul4) and RING finger protein 1 (ROC1) (Ito et al., 2010). Upon drug binding to Cereblon, specific substrate proteins are recruited to the E3 ligase and are targeted for ubiquitination and subsequent proteasomal degradation. The first such identified substrates, Ikaros and Aiolos, are lymphoid transcription factors that were found to be degraded to various extents in the presence of thalidomide, lenalidomide and pomalidomide in cell lines from various hematologic malignancies including multiple myeloma (MM), myelodysplastic syndrome (MDS), diffuse large B-cell lymphoma (DLBCL) and primary T cells (Gandhi et al., 2014; Gribben et al., 2015; Hagner et al., 2015; Kronke et al., 2015; Kronke et al., 2014; G. Lu et al., 2014). In MM cells treated with lenalidomide or pomalidomide, Aiolos and Ikaros degradation resulted in direct cell-autonomous effects including decreased proliferation which is associated with decreased levels of c-myc and interferon regulatory factor 4 (IRF4) (Bjorklund et al., 2015; Gang Lu et al., 2014). Lenalidomide

treatment of T and NK cells resulted in increased interleukin-2 (IL-2) and interferon-gamma (IFN- $\gamma$ ) secretion leading to enhanced immune mediated cytotoxicity including enhanced ADCC (Davies et al., 2001; Gandhi et al., 2014; Ramsay et al., 2008; Wu et al., 2008). Furthermore, Aiolos-deficient NK cells were shown to be strongly hyper-reactive in multiple tumor models (Holmes et al., 2014).

Despite the demonstrated clinical benefits of lenalidomide in R/R MCL and advances into the molecular mechanism in specific lymphoid malignancies, the MOA in MCL has remained largely uncharacterized. Here, we report that patients responding to lenalidomide therapy, but not non-responders, experienced significant increases in CD56<sup>+</sup> NK cells relative to total lymphocytes which was associated with a trend towards prolonged progression free survival and overall survival. Preclinical experiments reveal lenalidomide increased NK cell mediated cytotoxicity against MCL cell lines, primarily through enhanced formation of lytic NK cell immunological synapses and granzyme B secretion; compared to a lack in direct anti-proliferative effects against MCL cell lines. These data are consistent with a primarily immune-mediated mechanism of lenalidomide clinical activity in MCL.

## **Materials and Methods:**

### **MCL Cell Culture**

Mantle Cell Lymphoma (Jeko-1, JVM-2, Mino, Rec1, Granta-519, Z138) were obtained from American Type Culture Collection (ATCC) and were cultured in RPMI-1640 containing 10-20% fetal bovine serum, 1% Penicillin/Streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate.

### **Immunoblotting**

Cells were lysed in RIPA buffer containing 20 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 0.5 mM dithiothreitol, 1 mM

phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 2 µg/ml pepstatin. Proteins from cell lysates were separated by 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis (Bio-Rad), and transferred to nitrocellulose membranes (Invitrogen). Immunoblots were probed with antibodies recognizing: ITK (2F12) and phospho-BTK (Y223) (Cell Signaling). Signals were detected with a Li-Cor Odyssey imager.

#### **Isolation of PBMC and NK cells:**

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat (New York Blood Center) by Ficoll density gradient centrifugation. Natural killer (NK) cells were isolated by negative selection from PBMC according to manufacturer's protocol (Stemcell Tech). Isolated NK cell purity was over 85% as assessed by flow cytometry using anti-CD3 and anti-CD56 monoclonal antibodies (BD Biosciences).

#### **PBMC and NK cell co-culture experiments:**

PBMC and NK cells were treated with either DMSO, lenalidomide or ibrutinib for one hour prior to stimulation with either 3 µg/ml anti-CD3 (OKT3, eBiosciences) for 72 hours or 3 ng/ml IL-2 (R&D Systems) overnight, respectively. Prior to co-culturing with MCL cells, target cells were labeled with 200 nM of carboxyfluorescein succinimidyl ester (CFSE, Invitrogen). PBMC or NK cells were co-cultured with target cells at a defined ratio for an additional four hours. Subsequently, supernatant was collected for Granzyme B ELISA and apoptosis in labeled target cells was determined by flow cytometric analysis of Annexin V (BD Biosciences) and ToPro-3 (Invitrogen) according to manufacturer's protocol.

See supplemental experimental procedures for additional details.

## **Results:**

### **NK cells increase relative to total lymphocytes in patients with MCL who respond to lenalidomide therapy**

Lenalidomide has demonstrated clinically significant benefits in patients with R/R MCL; however, the molecular mechanism in MCL has not been described. In vitro studies have demonstrated lenalidomide has a dual MOA in other hematologic malignancies that includes both immunomodulatory effects on multiple immune cell types as well as cell autonomous apoptotic activity (Davies et al., 2001; Gandhi et al., 2014; Lopez-Girona et al., 2011; Mitsiades, 2002; Schafer et al., 2003; Wu et al., 2008). In a post-hoc analysis of biomarker data collected in the CC-5013-MCL-002 trial, we sought to examine the immunomodulatory effects of lenalidomide using samples collected from patients who had been treated with either lenalidomide or investigator's choice (single agent rituximab, gemcitabine, fludarabine, chlorambucil, or cytarabine) (Trněný et al., 2016). Peripheral blood samples were collected at Cycle 1 Day 1 (C1D1, pre-treatment), Cycle 1 Day 4 (C1D4), Cycle 2 Day 15 (C2D15). Flow cytometric profiling of T-cell (CD3 / CD4 / CD8 / CD45RA / CD25 / CD27), B-cell (CD19 / CD20 / CD5 / CD23 / CD40 / CD22 / Kappa / Lambda), and NK-cell (CD56 / CD16 / CD3 / CD69) subsets was performed. In baseline measurements of B, T and NK cells (total or subset populations), no significant differences were observed when comparing non-responder (stable or progressive disease) and responder (complete or partial response) outcome sub-groups in either lenalidomide (N=50) or investigator's choice control (N=18) arms (data not shown). However, we detected significantly elevated ( $p \leq 0.05$ ) levels of NK cells, as measured by CD3<sup>+</sup>CD56<sup>+</sup>CD16<sup>+</sup>, relative to total lymphocytes in patients who responded to lenalidomide (N=20, black dots) at both C1D4 and C2D15. Most importantly, this increase at C1D4 was also significantly different between responders compared to non-responders (N=14, gray dots) in the lenalidomide arm (Fig 1A). These observations were independent of baseline demographics such as age (71 versus 64),



number of prior therapies (2 vs 1) and ECOG score (1 vs 1) in responders compared to non-responders. Additionally, no differences in NK cell levels relative to total lymphocytes were observed in either responders or non-responders within the investigator's choice single agent arm. We confirmed our clinical observations with pre-clinical experiments, where treatment with lenalidomide increased CD56<sup>+</sup>CD16<sup>+</sup> NK cells in proportion to total lymphocytes in a CD3-stimulated peripheral blood mononuclear cell (PBMC) *in vitro* model with flow cytometric analysis (data not shown). Importantly, lenalidomide treated patients whose CD56<sup>+</sup>CD16<sup>+</sup> NK cells expanded above the median value of 9.8% observed at C1D4 demonstrated a trend towards prolonged progression free survival (PFS) (median of 48.7 weeks vs 16.9 weeks,  $p=0.11$ ) (Fig 1B). Additionally, there was a trend towards enhanced overall survival (OS) (median of undefined vs 86.7 weeks,  $p=0.08$ ) (Fig 1C). Taken together, these data provide a potential pharmacodynamic marker for response to lenalidomide in patients with R/R MCL and suggest that a lenalidomide-dependent increase in NK cells may contribute in part to extended PFS and OS.

#### **Clinical response to lenalidomide is not associated with baseline tumor microenvironment cereblon expression nor with mutations reported to impact response to ibrutinib**

We investigated the association of cereblon protein levels with clinical response by analyzing lymph node biopsies from R/R MCL patients enrolled in MCL-002. As shown in representative images in Fig 2A, cereblon was expressed at similar levels in patients with differing responses to therapy and quantitative H-scores for cereblon protein levels in total, nuclear, and cytoplasmic compartments did not correlate with clinical response to lenalidomide (Fig 2B). Previous studies have demonstrated that mutations in epigenetic regulators, mTOR signaling and the Bruton's tyrosine kinase (BTK) pathway, specifically *CREBBP*, *CD79A*, *ERBB4*, *MLL2*, *MTOR*, *PLCG2*, and *WHSC1* genes negatively correlate with clinical response to ibrutinib in MCL.(Balasubramanian et al., 2014; Lenz, 2016) To determine if mutations in these seven genes

influenced response to lenalidomide, we sequenced 19 patients (7 responders and 12 non-responders) from the MCL-002 trial using the Foundation One Heme panel. Our analysis suggested that lenalidomide retains activity in MCL patients whose tumors contain mutations that are associated with ibrutinib resistance, specifically *MLL2* where 7 patients achieved clinical outcomes of 4 responders and 3 non-responders (Fig 2C).

### **Lenalidomide increases immune cell-mediated cytotoxicity against MCL cell lines while ibrutinib does not**

To further investigate the immunomodulatory effects of lenalidomide we utilized an *in vitro* co-culture cytotoxicity assay. CD3-stimulated PBMCs were treated with DMSO or 1 nM to 10  $\mu$ M lenalidomide for 3 days prior to co-culture with CFSE labeled MCL cell lines. Increasing concentration of lenalidomide resulted in a 3 to 6.9-fold increase in CD3-stimulated immune cell-mediated cell death in Jeko-1, Granta-519, and Mino MCL cell lines as measured by Annexin V and ToPro-3 staining. Treatment with 10  $\mu$ M lenalidomide was significantly more effective at inducing PBMC-mediated apoptosis in all three MCL cell lines as compared to DMSO controls ( $p \leq 0.05$  Jeko-1,  $p \leq 0.0001$  Granta-519,  $p \leq 0.001$  Mino) (Fig 3A, left panel). In contrast, treatment with 10 nM to 1  $\mu$ M ibrutinib for 3 days prior to a four-hour co-culture with the same three MCL cell lines revealed no significant difference in induction of apoptosis compared to DMSO treated PBMCs (Fig 3A, right panel). In addition, analysis of the supernatant from these co-culture assays revealed that lenalidomide treatment increased secreted granzyme B levels in a dose dependent manner compared to vehicle controls (Fig 3B, left panel); however, treatment with ibrutinib did not result in increased granzyme B secretion (Fig 3B, right panel). Together, the functional cytotoxicity assays buttress the ability of lenalidomide to potentiate immune-mediated killing of MCL tumor cells and reveal granzyme B as a potentially important immunologic marker.

Given the duality of lenalidomide's MOA in other disease settings such as MM and DLBCL, we next examined the cell autonomous effects of lenalidomide in MCL. We first examined whether treatment with lenalidomide (0.01 nM to 10  $\mu$ M) impacted tumor cell proliferation by screening a panel of six MCL cell lines (Granta-519, Jeko-1, Mino, JVM-2, Rec-1, and Z138) for 5 days. Surprisingly, treatment with lenalidomide did not result in any significant changes in cellular proliferation as measured by  $^3$ H-thymidine incorporation (Fig S1A) or apoptosis in any of the MCL cell lines tested (Fig S1B). In contrast to lenalidomide, MCL cells treated with ibrutinib (0.01 nM to 10  $\mu$ M) or doxorubicin (150 nM to 10  $\mu$ M) for 5 days resulted in decreased proliferation (Fig S1C,D).

### **Lenalidomide enhances NK cell-mediated cytotoxicity and lytic immune synapse formation**

To determine whether NK cells were responsible for the ability of lenalidomide to promote immune-mediated cytotoxicity against MCL tumor cells, we utilized our *in vitro* co-culture cytotoxicity assay using negatively isolated CD56<sup>+</sup> NK cells. Interleukin-2 (IL-2) stimulated NK cells treated with 1 nM to 10  $\mu$ M lenalidomide for 18 hours prior to the co-culture with MCL cells resulted in remarkably similar anti-tumor activity to that observed in the previous co-culture assay using PBMCs (Fig 4A). Lenalidomide-treated NK cells were significantly more effective at inducing NK cell-mediated apoptosis of all three MCL cell lines when compared to vehicle treated cells ( $p \leq 0.01$  Jeko-1,  $p \leq 0.01$  Granta-519,  $p \leq 0.01$  Mino) (Fig 4A). In contrast, treatment with 0.5  $\mu$ M ibrutinib resulted in significantly reduced levels of apoptosis as measured by Annexin V/ToPro-3 staining in all 3 MCL cell lines ( $p \leq 0.0001$  Jeko-1,  $p \leq 0.01$  Granta-519,  $p \leq 0.0001$  Mino) compared to vehicle treated NK cells (data not shown). Moreover, supernatants from lenalidomide-treated co-cultures of NK cells with MCL cells revealed significantly increased granzyme B levels when compared to vehicle controls ( $p \leq 0.0001$  Jeko-1,  $p \leq 0.01$  Granta-519) (Fig 4B). Supernatants from ibrutinib-treated NK cells revealed significantly decreased levels of granzyme B compared to

vehicle control treatment ( $p \leq 0.0001$  Jeko-1 and Granta-519) suggesting that ibrutinib inhibits NK cell-mediated cytotoxicity (Fig 4B). The tyrosine kinase IL-2 inducible T cell kinase (ITK), has been shown to positively regulate FcγRIII, (also known as CD16) mediated NK cell cytotoxicity and ADCC (Khurana, Arneson, Schoon, Dick, & Leibson, 2007). Given the homology between BTK and ITK, we explored ibrutinib treatment on ITK activation in CD16 cross-linked NK cells. Ibrutinib resulted in decreased phosphorylation at tyrosine 180 (Y180) of ITK, confirming ITK as a known target of ibrutinib (Fig 4C) (Dubovsky et al., 2013). These data are consistent with previous reports indicating that ibrutinib antagonized rituximab-dependent NK cell-mediated cytotoxicity (Kohrt et al., 2014; Rajasekaran et al., 2014; Roit et al., 2014), however one group has demonstrated that ibrutinib increased NK cell-mediated activity of mouse NK cells (Kuo et al., 2016).

The addition of anti-CD20 monoclonal antibodies such as rituximab and obinutuzumab (GA101) to standard therapies have significantly improved responses in multiple hematologic malignancies (Coiffier et al., 2002; Goede et al., 2014). Lenalidomide has been shown to enhance ADCC and has demonstrated significant clinical benefit in non-Hodgkin's lymphoma (NHL). (Fowler et al., 2014; Hernandez-Ilizaliturri et al., 2005; Nowakowski et al., 2014; Ruan et al., 2015; Wang et al., 2012; Wu et al., 2008; L. Zhang et al., 2009) We sought to investigate the effect of lenalidomide in combination with CD20 targeting antibodies in a PBMC co-culture model. Expression of CD20 was confirmed via flow cytometry in Z138, JVM-2, and Granta-519 cell lines (Fig S2A). CD3 stimulated PBMCs were treated with lenalidomide (0.1  $\mu$ M or 1  $\mu$ M) for 3 days prior to four-hour co-culture with Granta-519, JVM-2, or Z138 cells that had been labeled with rituximab or obinutuzumab. Treatment with lenalidomide enhanced rituximab and obinutuzumab ADCC against all three MCL cell lines (Fig 4D, Fig S2B). Interestingly, the combination of lenalidomide with obinutuzumab was more potent than the combination with rituximab in all three cell lines, resulting in approximately 30% of MCL cells remaining viable following only four hours of co-culture.

Conditional deletion of Aiolos from NK cell lineages has been previously reported to result in NK cells that were hyper-reactive against tumor cells.(Holmes et al., 2014) We examined the effect of lenalidomide treatment on Aiolos and Ikaros protein expression in CD56<sup>+</sup> NK and CD3<sup>+</sup> T cells following treatment of PBMCs with increasing concentrations of lenalidomide (1 nM to 10  $\mu$ M) for 3 days compared to vehicle control. Our results showed that lenalidomide treatment decreased Aiolos and Ikaros levels in CD3<sup>+</sup> T cells, which is consistent with previous reports (Fig 4F).(Gandhi et al., 2014; Kronke et al., 2014) We also observed degradation of both Aiolos (40%) and Ikaros (95%) after lenalidomide treatment in CD56<sup>+</sup> NK cells (Fig S3A) suggesting that degradation of Aiolos and Ikaros may increase persistent NK cell activity against tumor cells.

We hypothesized that lenalidomide treatment could modulate the immunological synapse, which is the dynamic signaling interface formed between a NK cell and a target cell. Formation of the NK cell immune synapse controls the directed delivery of lytic granule contents for targeted cell lysis. F-actin polymerization is a hallmark of the activated NK cell lytic synapse, whereas the inhibitory immune synapse observed in NK cells is primarily actin-independent (Orange, 2008). Using confocal microscopy, we investigated the effects of lenalidomide on F-actin polymerization by visualizing NK:MCL cell conjugate interactions. Treatment of Jeko-1 and CD56<sup>+</sup> NK cells for 24 hours with 1  $\mu$ M lenalidomide prior to 30 minutes of co-culture resulted in increased F-actin polymerization as compared to DMSO control (Fig 4E). This increase in F-actin polymerization and focal expression pattern are characteristic of an immune synapse.(Davis & Dustin, 2004) Quantification of immune synapse formation in three independent experiments revealed that treatment with lenalidomide resulted in a statistically significant increase in immune synapse strength as measured by F-actin polymerization at the contact between a NK cell and a Jeko-1 cell ( $p \leq 0.0001$ ). Additionally, significant relocalization of perforin to the immune synapse ( $p \leq 0.0001$ ), a primary attribute of a functional lytic synapse, was observed (Fig 4F).

## Discussion:

Lenalidomide has significant activity in B-cell malignancies such as MM and NHL including DLBCL, FL and MCL.(Benboubker et al., 2014; Leonard et al., 2015; Trněný et al., 2016; Wiernik et al., 2008). To date, the mechanistic understanding of lenalidomide's anti-tumor activity was understood to be a combination of both direct cell autonomous and immune-mediated effects. In this study, we provide novel evidence that the clinical activity of lenalidomide in MCL is mediated through activation of NK cell directed cytotoxicity in patients. A subset analysis of longitudinal collected biomarker samples reveals that MCL patients enrolled on the MCL-002 trial who responded to lenalidomide had a significant expansion of CD56<sup>+</sup>CD16<sup>+</sup> NK cells relative to total lymphocytes compared to non-responders. Notably, this NK cell expansion was measurable as early as four days post-treatment initiation. Our correlative studies reveal that those patients whose NK cells expanded above the median increase for this population had a trend towards prolonged PFS and improved OS compared to patients whose NK cells expansion was below the median increase threshold. Interestingly, profiling of T cell subsets revealed a significant increase in CD4<sup>+</sup>CD25<sup>+</sup> activated T cells in lenalidomide treated patients at C2D15 compared to C1D1 (data not shown). However, this effect was not significantly different between responders and non-responders to lenalidomide. Further supporting the concept of an immune mediated anti-tumor process, lenalidomide exhibits activity in patients with a spectrum of lymphoma-associated mutations including those that have been correlated with disruption of ibrutinib activity in both MCL and CLL (Balasubramanian et al., 2014; Woyach et al., 2014), however these observations should be confirmed in a larger cohort.

While the precise molecular mechanism of lenalidomide's enhancement of NK cell activity is unknown, we describe a shared mechanism with T cells in that engagement of lenalidomide with CRL4<sup>CRBN</sup> results in decreased levels of the transcription factors Aiolos and Ikaros through ubiquitination and subsequent proteasomal degradation. Genetic deletion of Aiolos has been reported to significantly enhance NK mediated anti-tumor effects (Holmes et al., 2014).

Interestingly, lenalidomide treatment of NK cells has been shown to result in enhanced IFN-gamma secretion and actin remodeling as early as 90 minutes post-treatment, when degradation of Aiolos and Ikaros by CRL4<sup>CRBN</sup> is minimal (Laguerre, Carissey, Morgan, Chopra, & Davis, 2015). We hypothesize that the polymerization of F-actin and relocalization of perforin, two characteristics of an active immunological synapse, that were observed in our studies may be a distinct and independent mechanism from degradation of Aiolos and Ikaros and is currently under further investigation.

In conclusion, we believe these data identify a distinct immune-mediated mechanism of action for lenalidomide in MCL that should be confirmed in larger clinical studies. Lenalidomide treatment enhanced NK cell-mediated cytotoxicity by promoting lytic immune synapse formation and polarized granzyme B release. Furthermore, the improvement of anti-CD20 mediated ADCC following lenalidomide treatment provides pre-clinical mechanistic support for the combination of lenalidomide with anti-CD20 antibodies in MCL which has shown remarkable clinical activity in this disease setting with an overall response rate of 92%, complete response rate of 64% and progression free survival at 2 years of 85% (Ruan et al., 2015).

**Disclosure of conflict of interest: PRH, HC, MO, BA, MW, SC, MFW, EF, MT, AKG and AT are employed by and have equity ownership in Celgene Corporation. AGR receives research funding from Celgene. RC has equity ownership in Celgene Corporation.**

**Author contributions: PRH, HC, MO, MW, SC, MFW, AGR, MT, AKG and AT designed research, performed research and interpreted results. PRH, EF, AGR, AKG, RC and AT assisted with manuscript preparation.**

## Supporting information:

### Materials and Methods

Fig. S1. Cell autonomous effects of lenalidomide, ibrutinib and doxorubicin in MCL cell lines.

Fig. S2. Lenalidomide dependent antibody dependent cell-mediated cytotoxicity of Rituximab and Obinutuzumab labeled MCL cell lines.

Fig. S3. Lenalidomide treatment results in decreased levels of Aiolos and Ikaros.

### Figure Legends:

**Fig. 1. Examination of peripheral NK cells in patients treated with lenalidomide or investigators choice in MCL-002.** (A) Scatter plot of CD3<sup>+</sup>CD56<sup>+</sup>16<sup>+</sup> proportions to total lymphocytes collected at indicated time points and grouped by responder/non-responder for the lenalidomide and investigator's choice arm. \*: p<0.05 (B) Kaplan-Meier curves plotting the progression free survival of patients above (red line) or below (black line) the median increase in relative proportion of NK cells to lymphocytes. (C) Kaplan-Meier curves plotting the overall survival of patients above (red line) or below (black line) the median increase in relative proportion of NK cells to lymphocytes.

**Fig. 2. Lenalidomide activity in CC-5013-MCL-002 is independent of mutational status and cereblon protein levels.** (A) Representative fields of cereblon IHC on FFPE lymph node biopsies obtained from MCL-002 patients grouped by response to lenalidomide. (B) Total, nuclear and cytoplasmic cereblon were scored using an H-score system. Box and whisker plots for H-scores grouped by best overall response to lenalidomide. Progressive disease (PD), n=7; stable disease (SD), n=17; partial response (PR), n=21. (C) Graphical representation of mutational status for



*CREBBP*, *CD79A*, *ERBB4*, *MLL2*, *MTOR*, *PLCG2* and *WHSC1* compared to best overall response from biopsies (n=19) obtained prior to lenalidomide treatment.

**Fig. 3. Lenalidomide and ibrutinib dependent PBMC-mediated cytotoxicity against MCL cell lines.** (A) CD3 stimulated PBMCs treated with DMSO, lenalidomide (0-10  $\mu$ M), or ibrutinib (0-1  $\mu$ M) for 3 days, prior to a four hour co-culture with MCL cell lines (Granta-519, Jeko-1, and Mino). Apoptosis was analyzed by Annexin V and ToPro-3 staining. Data from 3 independent experiments are presented as percentage of viable cells versus DMSO control  $\pm$  standard error of the mean (SEM). (B) Supernatant from co-cultures of PBMC and MCL cells were analyzed for granzyme B by ELISA. Data are presented as fold change from DMSO  $\pm$  SEM.

**Fig. 4. Lenalidomide dependent NK cell mediated cytotoxicity and immune synapse formation** (A) IL-2 stimulated NK cells treated with DMSO or lenalidomide (0-10  $\mu$ M) for 18 hours, prior to a four hour co-culture with MCL cell lines (Granta-519, Jeko-1, and Mino). Apoptosis was analyzed by Annexin V and ToPro-3 staining. Data from 3 independent experiments are presented as percentage of viable cells versus DMSO control  $\pm$  SEM. (B) Supernatant from co-cultures of MCL cells and NK cells treated with DMSO, lenalidomide (1  $\mu$ M) and ibrutinib (500 nM) were analyzed for granzyme B by ELISA. Data from 3 independent experiments are presented as fold change from DMSO  $\pm$  standard deviation (SD). (C) IL-2 stimulated CD56+ NK cells treated with DMSO, lenalidomide or ibrutinib (0.1-1  $\mu$ M) for 18 hours prior to CD16 stimulation. Cell lysates were separated by SDS-PAGE, and levels of phospho-ITK (Y180) and ITK were assessed. (D) CD3 stimulated PBMCs treated with DMSO, lenalidomide (0.1-1  $\mu$ M) for 3 days, prior to a four hour co-culture with rituximab or obinutuzumab labeled Granta-519 cells. Apoptosis was analyzed by Annexin V and ToPro-3 staining. Data from 3 independent experiments are presented as percentage of viable cells versus DMSO  $\pm$  SEM. (E)

Representative fields of immune synapse formation between NK and Jeko-1 cells treated with DMSO or lenalidomide (1  $\mu$ M). F-actin staining via phalloidin incorporation (red) and perforin (green), MCL cells were labeled with CMAC (blue). (F) Polymerized F-actin (left panel) and perforin (right panel) localized to the immune synapse was quantified. Each data point represents a distinct immune synapse. Results of 3 independent experiments are shown. \*\*:  $p < 0.01$ , \*\*\*\*:  $p < 0.0001$

## References and Notes

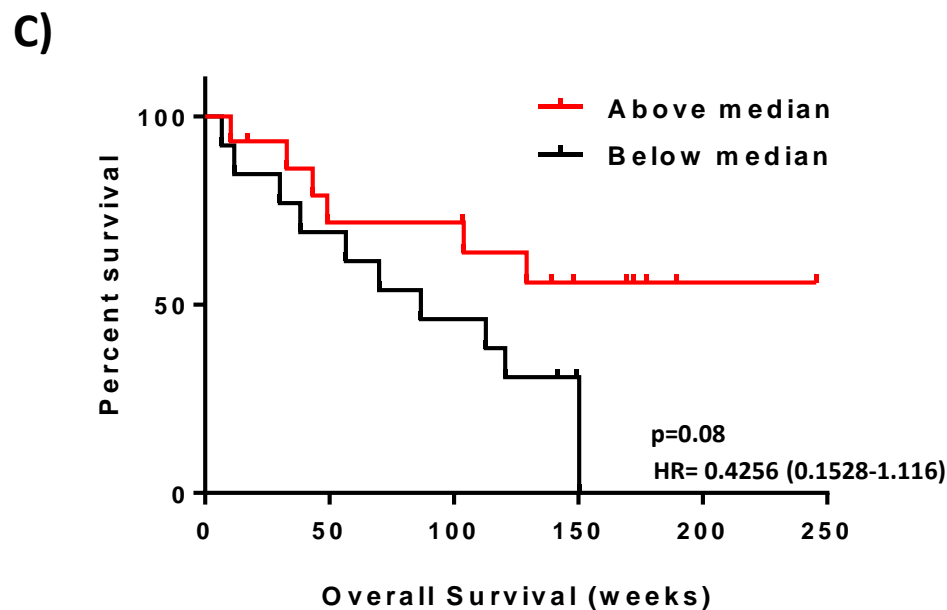
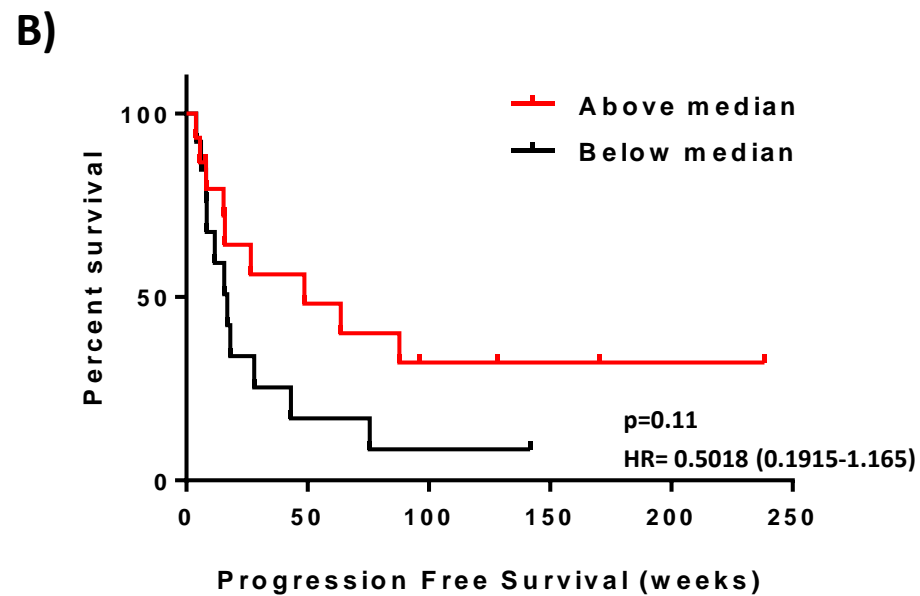
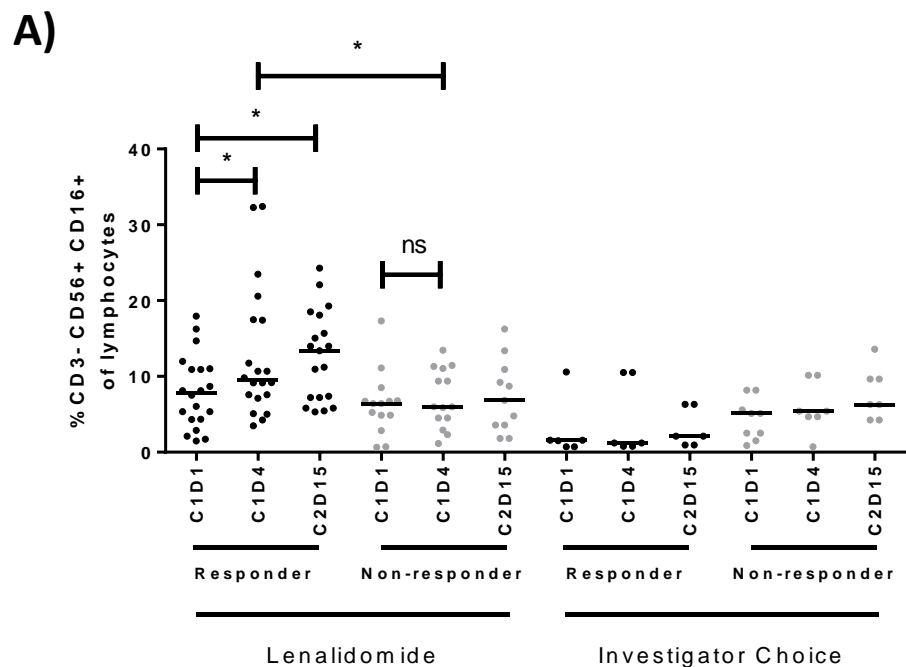
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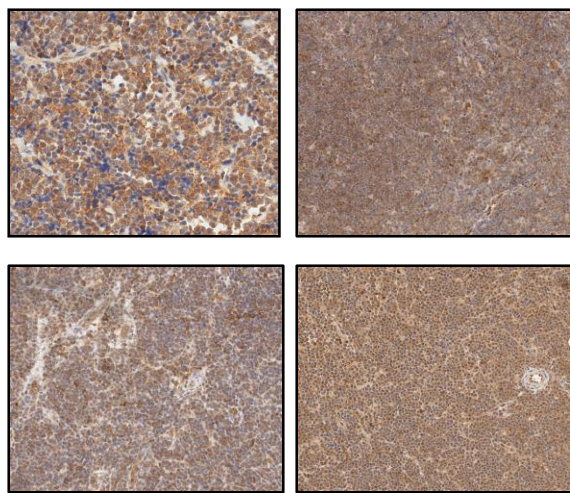
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**Figure 1**

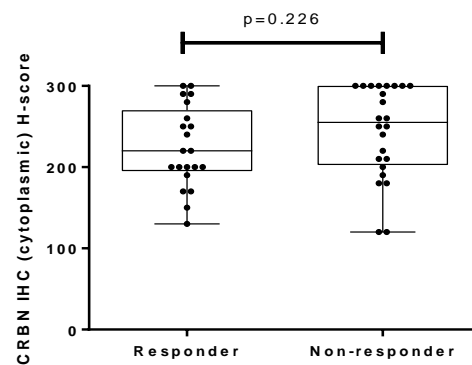
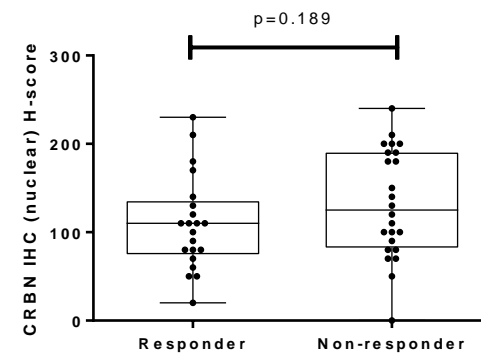
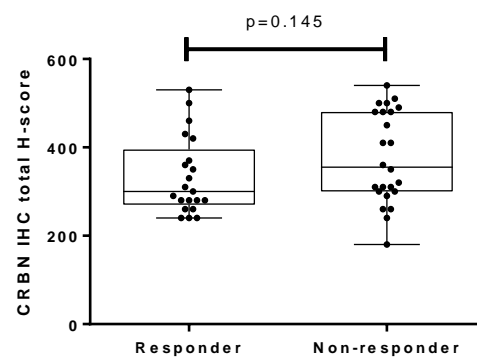
A)



Responder

Non-responder

B)



C)

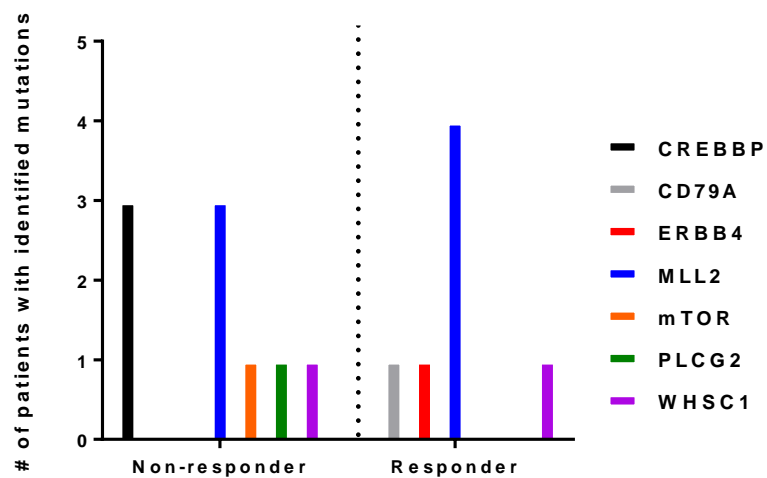
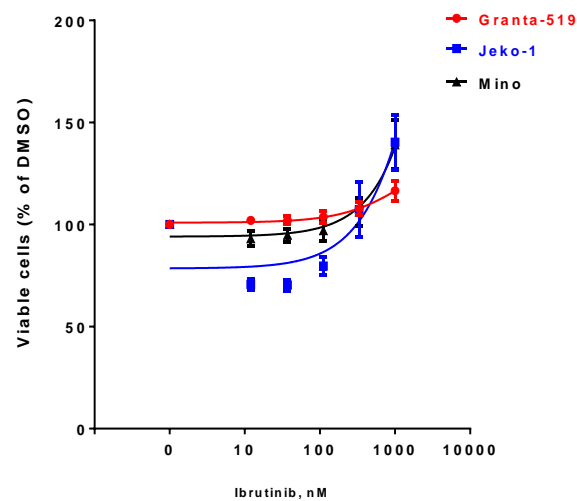
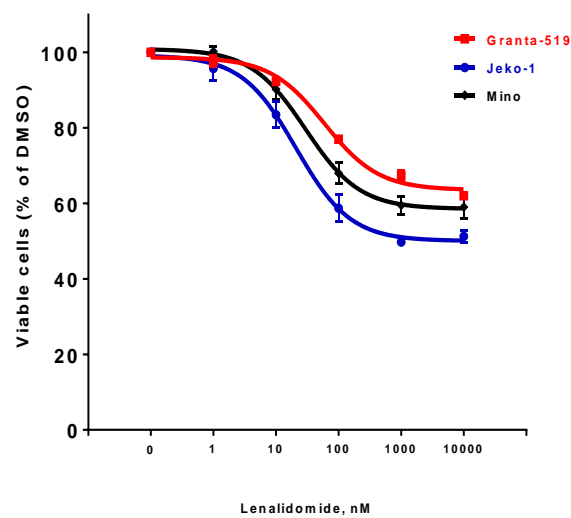


Figure 2



A)



B)

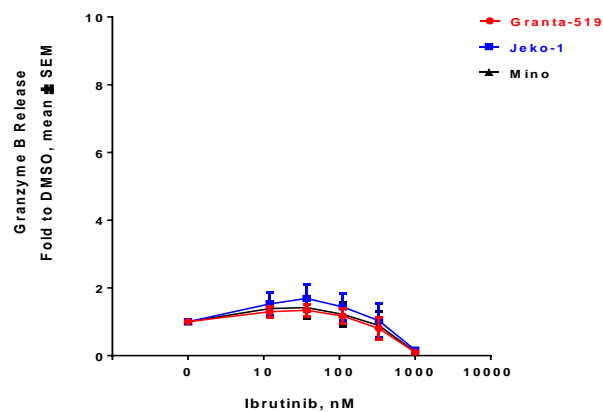
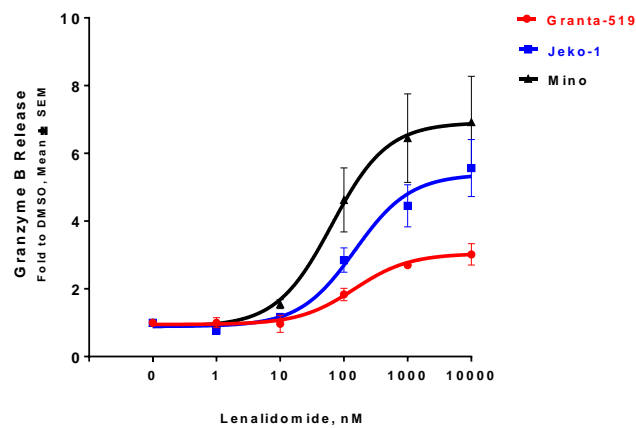
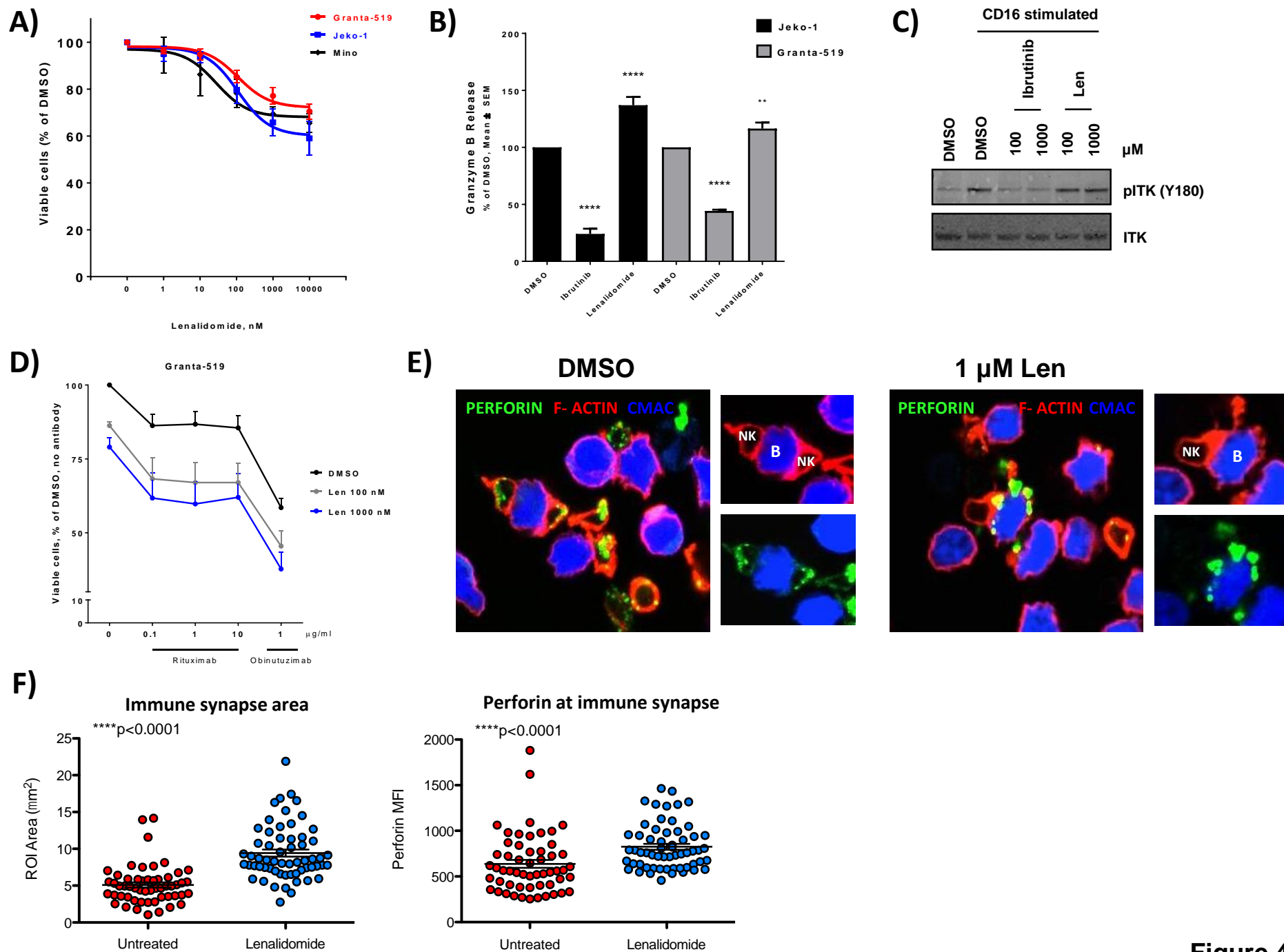


Figure 3



**Figure 4**

## **Supporting information:**

### Materials and Methods

Figure. S1. Cell autonomous effects of lenalidomide, ibrutinib and doxorubicin in MCL cell lines.

Figure. S2. Lenalidomide dependent antibody dependent cell-mediated cytotoxicity of Rituximab and Obinutuzumab labeled MCL cell lines.

Figure. S3. Lenalidomide treatment results in decreased levels of Aiolos and Ikaros.

## **Supplemental Materials and Methods**

### **Granzyme B ELISA:**

Supernatants harvested from co-culture experiment were examined at 1:5 dilution in complete media for secreted Granzyme B analysis according to manufacturer's protocol (Cell Science).

### **Flow Cytometry:**

MCL cells ( $1 \times 10^5$  cells/ml) were treated with either DMSO or various concentrations of lenalidomide for 7 days. Cells were collected and apoptosis was analyzed through flow cytometric analysis of Annexin V and To-Pro 3 staining according to manufacturer's protocol (Life Technologies). The gating strategy utilized for determining the quadrants in the flow cytometry data segregates the majority of viable cells in the DMSO control within each cell line as Annexin V negative/ ToPro-3 negative. BDFACS Fortessa flow cytometer equipped with FACSDIVA (Becton-Dickinson) were used for acquisition. Flowjo (Flowjo LLC) was used for analysis of exported FACS data.

### **Proliferation assays**

$2 \times 10^4$  cells were plated per well in media containing either DMSO or various concentrations of lenalidomide or ibrutinib. Cells were cultured for 5 days at 37 degrees Celsius after which tritiated thymidine was added to the cell culture for the final 6 hours. Cells were subsequently harvested onto filter plates. After the plates have dried, scintillation fluid was added to the plates and read on a Top-count reader (Perkin-Elmer).

### **Statistical Analysis:**

Significance among different groups were assessed using one-way ANOVA from Graphpad Prism (GraphPad Software, Inc.). Data were expressed as mean  $\pm$  SEM. In this study, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

### **Immune Synapse Immunofluorescence:**

Briefly, Jeko-1 MCL cells ( $2 \times 10^6$ ) were stained with CellTracker Blue CMAC according to manufacturer's instructions and pulsed with 2  $\mu\text{g/mL}$  of a cocktail of staphylococcal superantigens (sAgs; SEA and SEB; Sigma-Aldrich) for 30 minutes at 37°C. Jeko-1 cells were centrifuged at 200g for 5 minutes with an equal number of DMSO or Lenalidomide treated NK cells (purified from PBMC as described above) and incubated at 37°C for 15 minutes. Cells

were transferred onto microscope slides (Menzel-Glaser Polysine slides; Thermo Scientific) using a cell concentrator (Cytofuge 2) and fixed for 15 minutes at room temperature with 3% methanol-free formaldehyde (TABB Laboratories) in PBS. Immunofluorescent labeling was done using Cytofuge2 cell concentrator units. Cells were permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 5 minutes and treated for 10 minutes with 0.1% BSA in PBS blocking solution. Primary and secondary antibodies were applied sequentially for 45 minutes at 4°C in 5% goat serum (Sigma-Aldrich) in PBS blocking solution. F-actin was stained with rhodamine phalloidin and perforin was stained with (clone dG9, Biolegend) according to manufacturer's protocol. After washing, the cell specimens were sealed with 22- x 32-mm coverslips using fluorescent mounting medium (Dako). Medial optical section images were captured with a Zeiss 510 confocal laser-scanning microscope using a 63x/1.40 oil objective and LSM Version 3.2 SP2 imaging software (Zeiss). Detectors were set to detect an optimal signal below saturation limits. Fluorescence was acquired sequentially to prevent passage of fluorescence from other channels (Multi-Track). Image sets to be compared were acquired during the same session using identical acquisition settings. Blinded confocal images were analyzed using AxioVision Version 4.8 image analysis software (Zeiss). NK/Jeko-1 cell conjugates were identified only when NK cells were in direct contact interaction with Jeko-1 (blue fluorescent channel). The AxioVision area analysis tool was then used to measure the total area (in square micrometers) of F-actin (red fluorescent channel) accumulation at all NK-cell contact sites and synapses with Jeko-1 cells. These data were then exported into Prism Version 5 software (GraphPad) for statistical analysis and to generate a mean area value per experimental population.

#### **Foundation Medicine Mutational Analysis:**

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega) and quantified using the PicoGreen fluorescence assay (Invitrogen). Library construction was performed as previously described using 50–200 ng of DNA sheared by sonication to ~100–400 base pairs before end repair, dA addition and ligation of indexed Illumina sequencing adaptors. Enrichment of target sequences (3,320 exons of 182 cancer-related genes and 37 introns from 14 genes recurrently rearranged in cancer representing ~1.1 Mb of the human genome in total) was achieved by solution-based hybrid capture with a custom Agilent SureSelect biotinylated RNA baitset. The libraries were sequenced on an Illumina HiSeq 2,000 platform using 49 x 49 paired-end reads. Sequence data from genomic DNA was mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner and were processed using the publicly available SAMtools, Picard and

Genome Analysis Toolkit. Genomic rearrangements were detected by clustering chimeric reads mapped to targeted introns.

### **Immunohistochemistry**

Four micron thick FFPE tumor sections were stained with antibodies to cereblon (rabbit monoclonal antibody; Celgene CRBN65) using the Bond-Max automated slide strainer (Leica Microsystems, Buffalo Grove, IL) and the Bond Polymer Refine Detection Kit. Antigen retrieval was performed with Epitope Retrieval 2 (pH 9.0) for 20 min at 100°C on the instrument. The slides were blocked for endogenous peroxidase activity with Peroxide Block for 5 min at room temperature. Sections were then incubated with primary antibodies for 15 minutes at room temperature. Horseradish peroxidase (HRP) labelled Polymer was applied at the instrument's default conditions and diaminobenzidine tetrahydrochloride (DAB) was used as the enzyme substrate to visualize specific antibody localization. Slides were counterstained with hematoxylin. H-scores for cereblon were generated with a H Score =  $\sum(1+i)p_i$  where i is the intensity score and  $p_i$  is the percent of the cells with the corresponding intensity.

### **CC-5013-MCL-002 clinical study and immunohistochemistry**

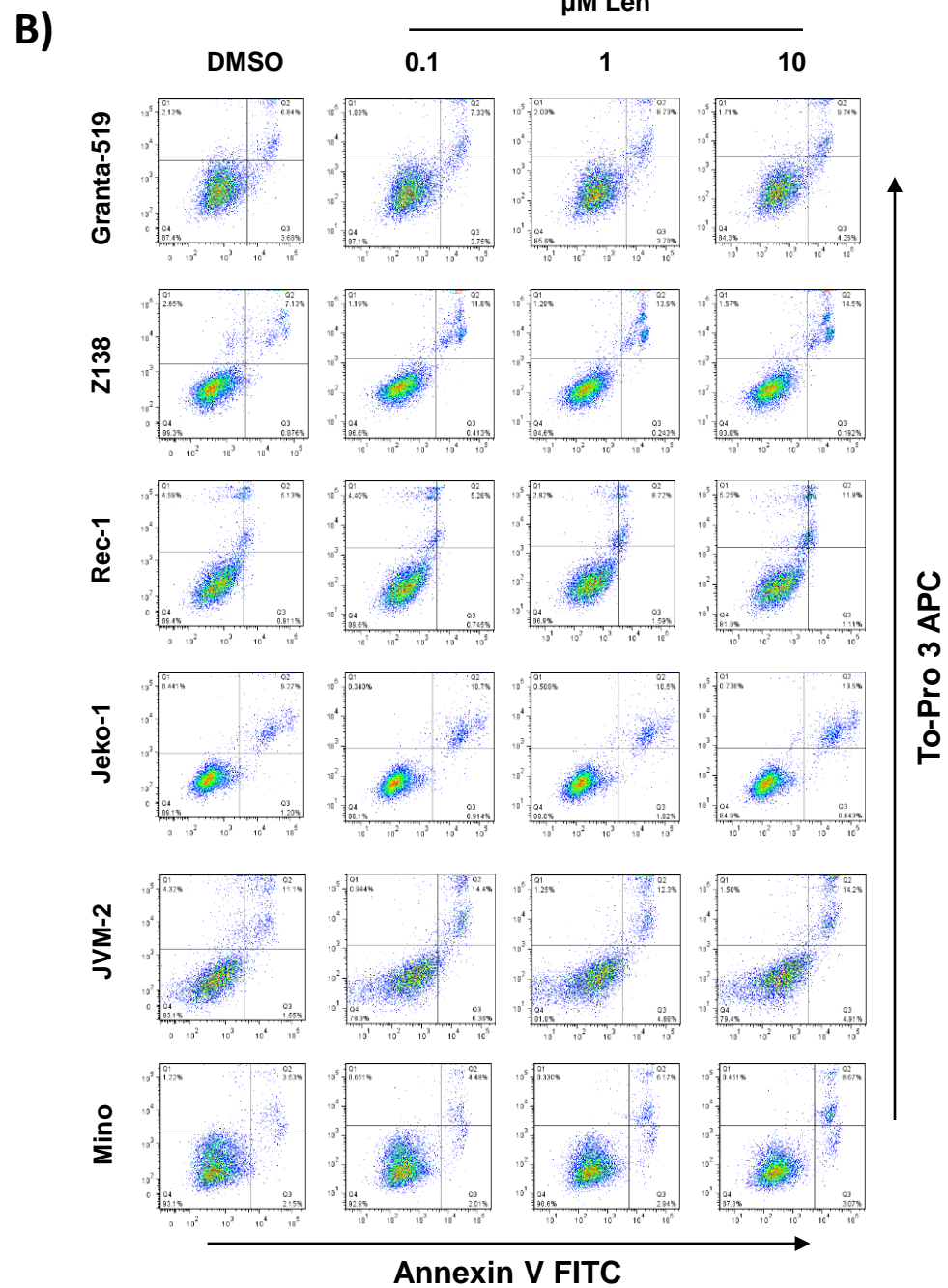
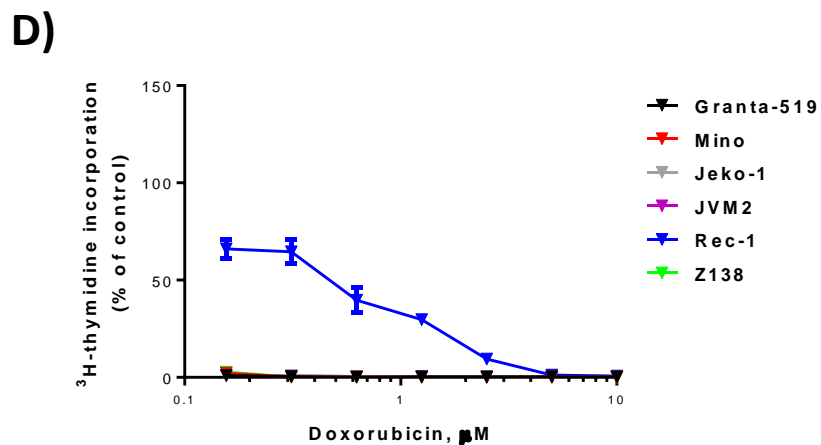
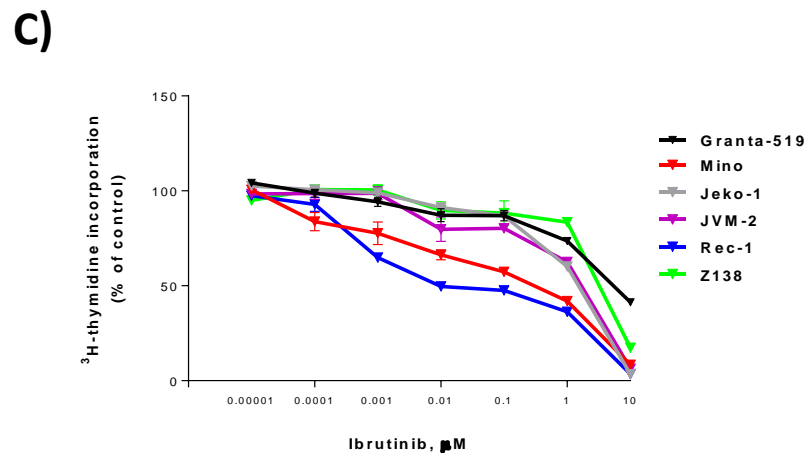
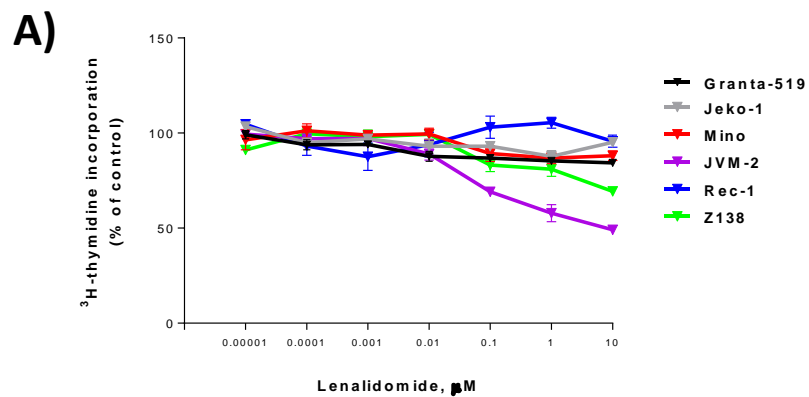
The study protocol and informed consent form were approved by the institutional review board/independent ethics committees of participating institutions. Written informed consent was obtained from all participants, and the trial was conducted in accordance with the Helsinki declaration. Immunohistochemistry for cereblon was performed on FFPE samples as described above.

### **Supplemental Figure Legends:**

**Figure S1: Cell autonomous effects of lenalidomide and ibrutinib in MCL cell lines.** A) MCL cell lines were treated with DMSO or lenalidomide (0.001 nM to 10  $\mu$ M) for 5 days. Proliferation was determined using  $^3$ H-thymidine incorporation. Results of three independent experiments are shown  $\pm$  SEM. B) MCL cell lines were treated with DMSO or lenalidomide (0.1-10  $\mu$ M) for 7 days, after which apoptosis was measured by Annexin V and To-Pro 3 flow cytometric analysis. C) MCL cell lines were treated with DMSO or ibrutinib (0.001 nM to 10  $\mu$ M) for 5 days. Proliferation was determined using  $^3$ H-thymidine incorporation. Results of three independent experiments are shown  $\pm$  SEM.

**Figure S2: Lenalidomide dependent antibody dependent cell-mediated cytotoxicity of Rituximab and Obinutuzumab labeled MCL cell lines.** A) Flow cytometry analysis of CD20 expression levels on MCL cell lines. Histograms show labeling of CD20 (blue histogram) or with a mouse isotype control antibody (red histogram). OCI-LY10 (DLBCL cell line) and NK92 (NK cell line) serve as positive and negative controls, respectively. B) Anti-CD3 stimulated PBMCs treated with DMSO, lenalidomide (0.1-1  $\mu$ M) for 3 days, prior to a four hour co-culture with Rituximab or Obinutuzumab labeled JVM-2 and Z138 cell lines. Apoptosis was analyzed by Annexin V and ToPro-3 staining. Data from 3 independent experiments are presented as percentage of viable cells versus DMSO control  $\pm$  SEM.

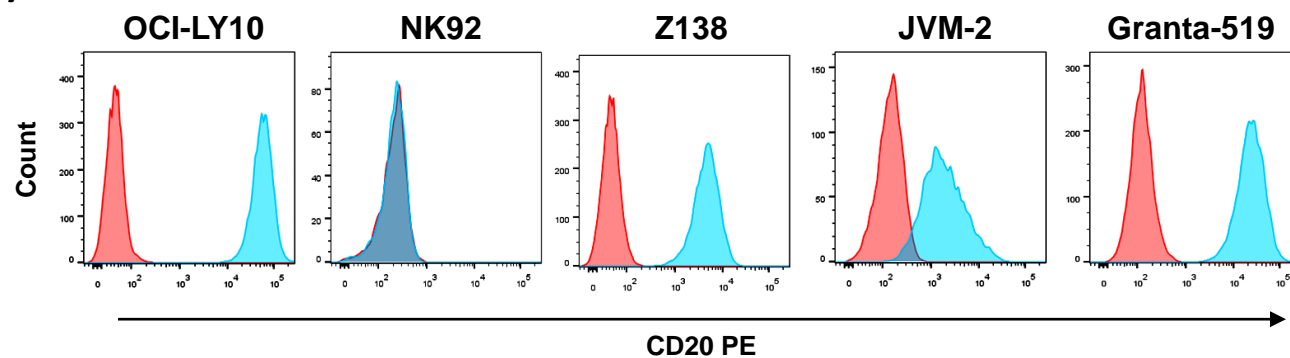
**Figure S3: Lenalidomide treatment results in decreased levels of Aiolos and Ikaros.** (A) Flow cytometric analysis of intracellular Aiolos and Ikaros levels in NK and T cells treated with DMSO or lenalidomide 0-10  $\mu$ M). Data from 3 independent experiments are presented as fold change from DMSO  $\pm$  SEM.



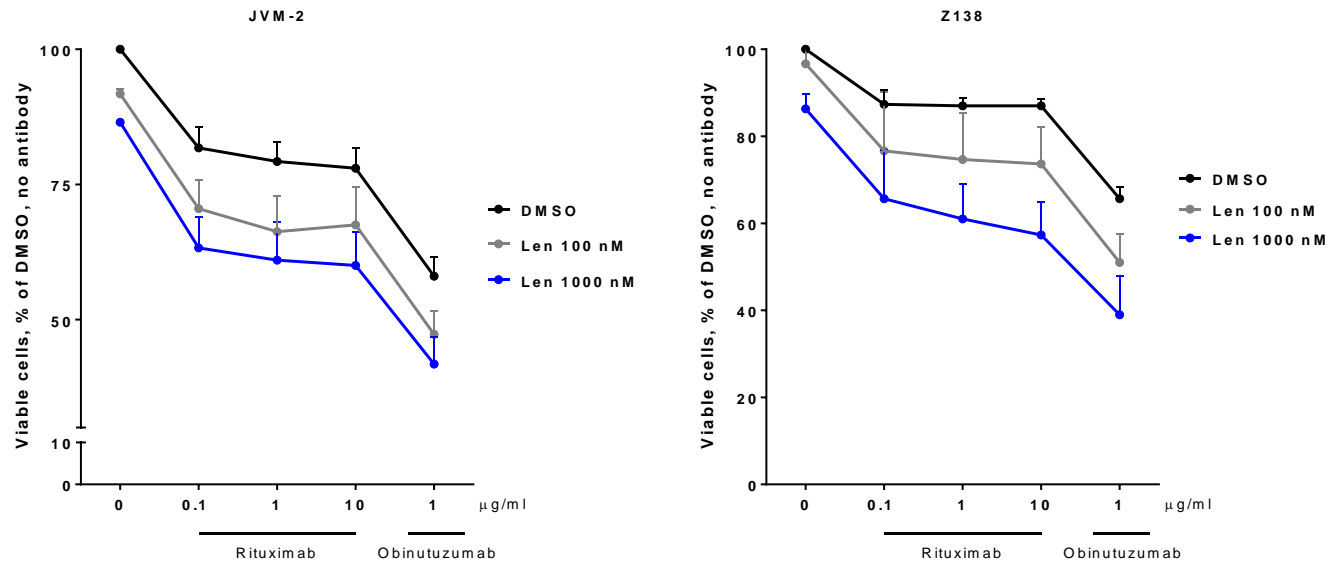
**Fig S1**



**A)**



**B)**



A)

